within the early region that function weakly in the absence of *rho*. Although it has been demonstrated that both the T3 and T7 phage genomes contain *rho*-dependent transcription termination sites in the early region (Adhya et al., 1979; Darlix & Horaist, 1975; Kassavetis & Chamberlin, 1981), in a preliminary study we have found that low concentrations of the *E. coli rho* factor affected only the $\phi$Cd1 in vitro transcripts A′ and A. Alternatively, termination could result from discrete RNA polymerase pause sites similar to those that have been found in the early region of T7 DNA under suboptimal substrate concentrations (Darlix & Horaist, 1975; Kassavetis & Chamberlin, 1981). It is unlikely, however, that the B and C transcripts observed in this study are due to pause-site termination since synthesis occurred in the presence of excess nucleoside triphosphate (0.5 mM and labeled nucleotide at 0.1 mM) and could not be chased into higher molecular weight transcripts once they were completed. The possibility remains that there are weak termination sites within the early region of the $\phi$Cd1 genome that function to prevent complete read through of some enzyme molecules. These sites could be structurally similar to but independent of RNase III sites and because of the high G+C content could function differently with the *C. crescentus* RNA polymerase and the *E. coli* RNA polymerase or *rho* factor. In fact, we have recently demonstrated that although the *E. coli* RNA polymerase synthesizes the major A transcript from the $\phi$Cd1 DNA in vitro, the C and D transcripts are replaced by transcripts of different sizes.

Acknowledgments

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# Enzymatic Synthesis of a 21-Nucleotide Coat Protein Binding Fragment of R17 Ribonucleic Acid†

Marc Krug, Pieter L. de Haseth,‡ and Olke C. Uhlenbeck*

ABSTRACT: An oligoribonucleotide with a sequence identical with the bacteriophage R17 replicase initiator region has been synthesized. The sequence also encompasses the binding domain of R17 coat protein, which is known to act as a translational repressor at this site. The 21-nucleotide fragment was synthesized entirely by enzymatic methods, T4 RNA ligase being used to join shorter oligomers. The resulting fragment has a secondary structure with the expected thermal stability. Since the synthetic fragment binds R17 coat protein with the same affinity as a 59-nucleotide fragment isolated from R17 RNA, we conclude that it has full biological activity.

The specific "recognition" of an RNA sequence by a protein is thought to be due to a number of specific contacts spread over the surface of both molecules. A promising approach toward the identification of these contacts has been to use an in vitro synthesized target sequence that can be modified at will. For example, this approach has been used to examine the details of the interactions between *lac* repressor and *lac* operator DNA (Caruthers, 1980). In this paper we describe the synthesis of a short RNA fragment that is involved in a specific RNA–protein interaction. The procedure permits the construction of variant RNA fragments, which should allow the identification of the nucleotides in contact with the protein.

About 10 min after infection, translation of the bacteriophage R17 replicase gene is repressed when the product of the coat protein gene binds to the replicase initiator region (Sugiyama & Nakada, 1968). This binding is specific and strong enough to permit the protection of a 59-nucleotide fragment from ribonuclease $T_1$ digestion of the genomic RNA (Bernardi

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received April 14, 1982.* This work was supported by a grant from the National Institutes of Health (GM 19059).

‡ Present address: Department of Chemistry, University of Colorado, Boulder, CO 80309.

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& Spahr, 1972). This fragment, designated R(−53 → +6) where +1 is the first nucleotide of the replicase gene, can effectively rebind the coat protein with an affinity similar to intact RNA (Berzin et al., 1978). The coat protein binding site on the RNA has been shown by thermal relaxation (Gralla et al., 1974) and NMR[1] (Hilbers et al., 1974) studies to involve the smaller of the two hairpin helices that comprise the region R(−15 → +4). Furthermore, the replicase initiator region R(−17 → +11) isolated by a ribosome protection experiment can also rebind the coat protein (Steitz, 1974). More recent coat protein selection experiments (V. Cameron, J. Carey, P. L. de Haseth, and O. C. Uhlenbeck, unpublished results) also locate the binding site to the small hairpin helix.

This well-documented case of translation repression provides an excellent example of a highly specific RNA–protein interaction that can be described by a simple binding equilibrium. Since effective rebinding of the 59-nucleotide fragment to the coat protein occurs, all the contacts between protein and nucleic acid appear to exist within this fragment. In this paper we describe the synthesis of a 21-nucleotide fragment corresponding to R(−17 → +4) that should encompass the entire coat protein binding site. The synthesis is totally enzymatic, employing polynucleotide phosphorylase to prepare short oligomers (Thach & Doty, 1965) and T4 RNA ligase and polynucleotide kinase to join them (Gumport & Uhlenbeck, 1981). We will demonstrate that the synthetic fragment has the expected secondary structure and is capable of binding R17 coat protein with full activity.

## Experimental Procedures

### *Materials*

Dinucleoside phosphates, nucleoside diphosphates, and ATP were purchased from Sigma Chemical Co. Nucleoside 3′-(2′),5′-bisphosphates were prepared according to Barrio et al. (1978). Tritium-labeled nucleoside diphosphates (9–18 Ci/mmol) were purchased from Amersham or Nuclear Dynamics. [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) was prepared from $^{32}$P-labeled inorganic phosphate (Johnson & Walseth, 1979).

T4 RNA ligase (sp act. 2100 units/mg) was purified by the method of Moseman-McCoy et al. (1979). Polynucleotide kinase was purified from T4 wild-type (Cameron & Uhlenbeck, 1977) and *PseT* 1 (Soltis & Uhlenbeck, 1982) infected cells. Primer-dependent polynucleotide phosphorylase from *Micrococcus luteus* (50 units/mg) was purified by a modification of the method of Klee (1971). Ribonucleases A, $T_1$, and $T_2$ were purchased from Calbiochem, *Escherichia coli* alkaline phosphatase was from Worthington Biochemical, and creatine phosphokinase was from Sigma Chemical Co.

Isolation of R17 coat protein and the 59-nucleotide coat protein binding fragments from R17 RNA will be described elsewhere (V. Cameron, J. Carey, P. L. de Haseth, and O. C. Uhlenbeck, unpublished results). That paper also will describe in detail the binding assay utilized to measure the association of coat protein with the natural or synthetic fragments.

### *Methods*

Descending paper chromatography of oligoribonucleotides was carried out on Whatman No. 3 MM paper with one of the following v/v mixtures of 95% ethanol and 1 M ammonium acetate: solvent A (60:40), solvent B (50:50), or solvent C (30:70). Oligomers were located on the chromatogram by viewing with ultraviolet light or by cutting the paper in 1-cm sections and counting them in a liquid scintillation counter. Recovery of oligomers from paper is described in England & Uhlenbeck (1978).

Column chromatography of oligoribonucleotides was carried out with small (3–10-mL) DEAE-Sephadex A25 columns. Oligomers were eluted with a linear gradient of 0.05–0.75 M triethylammonium bicarbonate (TEAB), pH 7.5. The total gradient volume was generally about 40 column volumes. Each peak was desalted by rotary evaporation aided by the addition of methanol. Oligomers were subsequently desalted on a Bio-Gel P2 or Sephadex G10 column equilibrated in neutralized water. The second desalting step was required to remove minor impurities that inhibited enzyme reactions.

Longer oligonucleotides were separated on 20% polyacrylamide gels containing 7 M urea in 0.1 M Tris–borate, pH 8.1 (Donis-Keller et al., 1977). Electrophoresis was at 800 V for 2.5 h. Analytical slab gels (270 × 170 × 0.5 mm) were stained with Stains-All (Dahlberg et al., 1969) or autoradiographed to locate oligomers. The same type of gel was used to purify the 21-nucleotide coat binding fragment from the final RNA ligase reaction. Oligomers were eluted from gels by crushing the gel slice and soaking it overnight in 1.5 mL of 0.5 M sodium acetate (pH 5.3), 1 mM EDTA, and 0.1% $NaDodSO_4$ at 4 °C. After centrifugation through a 3-mL polypropylene column with paper filter (Biolab Products) to remove acrylamide fragments, 3 volumes of ethanol were added. In cases where the oligomers were short or in very low concentrations, $MgCl_2$ was added to 1 mM before the ethanol was added. After 4 h at −60 °C, the precipitated oligomer was collected by centrifugation at 1000*g* for 10–20 min and redissolved in distilled water.

$^{32}$P-Labeled oligonucleotides were hydrolyzed to give 3′ mononucleotides by incubating them with 40 units/mL ribonuclease $T_2$, 50 units/mL ribonuclease $T_1$, and 10 $\mu$g/mL ribonuclease A in 50 mM ammonium acetate, pH 4.5, for 2 h at 37 °C. $^{32}$P-Labeled oligonucleotides were hydrolyzed to leave 5′ mononucleotides by incubating with 1 unit/mL snake venom phosphodiesterase, 130 $\mu$M $(pU)_4$ carrier, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 8.5, for 2 h at 37 °C. The mononucleotides were separated on two-dimensional cellulose thin-layer plates as described by Nishimura (1979) and located by autoradiography.

*Oligoribonucleotide Synthesis.* Two standard buffers were used in all oligomer reactions. Buffer A was used for polynucleotide phosphorylase reactions and contains 10 mM $MgCl_2$, 0.4 M NaCl, and 0.2 M Tris-HCl, pH 8.2. Buffer B was used for RNA ligase reactions and contains 20 mM $MgCl_2$, 3 mM dithiothreitol, and 50 mM Hepes at the pH indicated.

$(Ap)_3C$ was prepared as described previously (Uhlenbeck & Cameron, 1977). $UpA(pC)_3$ was synthesized from UpA and CDP with polynucleotide phosphorylase. A 1.1-mL reaction contained 7.4 mM UpA, 46 mM CDP, buffer A, and 7.7 units/mL polynucleotide phosphorylase. After incubation for 72 h at 37 °C, the reaction was terminated by heating to 85 °C for 5 min. The reaction was diluted to 3.3 mL with water and incubated with 0.1 mg/mL bacterial alkaline phosphatase for 4 h at 37 °C to degrade unreacted CDP. The reaction was further diluted and purified on a 10-mL Sepadex A-25 column. The dimer and trimer peaks were identified by their comigration with standards on paper chromatography in solvent A. The amount of the pentamer $UpA(pC)_3$ re-

[1] Abbreviations: NMR, nuclear magnetic resonance; ATP, adenosine 5′-triphosphate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

covered (0.8 μmol) corresponds to a 10% yield with respect to the input dimer.

ApUpG and CpApG were prepared by a ribonuclease $T_1$ assisted polynucleotide phosphorylase reaction. Each 5-mL reaction contained 5 mM dimer, 20 mM GDP, 40 μg/mL ribonuclease $T_1$, buffer A, and 4.0 units/mL polynucleotide phosphorylase. After 48 h at 37 °C, the reactions were heated to 85 °C for 5 min, diluted to 15 mL with water, and incubated with 0.1 mg/mL bacterial alkaline phosphatase at 37 °C for 4 h. Purification of the trimers was accomplished on a 3-mL DEAE-Sephadex column. Recovery of ApUpG (21 μmol) and CpApG (16 μmol) corresponds to 85 and 65% yields, respectively.

The preparation of pApUpGp was identical with that of ApUpG up to the heat inactivation of the polynucleotide phosphorylase. At this point the reaction mixture was applied directly to a 150-mL [*N*-[*N'*-[*m*-(dihydroxyboryl)phenyl]-succinamyl]amino]ethylcellulose (DBAE-cellulose) column (Weith et al., 1970) equilibrated in 1.0 M TEAB, pH 8.5, and eluted with the same buffer. A mixture of ApUpGp and GMP flowed directly through the column while GDP was retarded. This step is necessary since GDP and ApUpGp coelute with DEAE-Sephadex. The ApUpGp was separated from GMP by diluting the fraction to 0.15 M TEAB and applying it to a 5-mL DEAE-Sephadex A-25 column, washing with the same buffer to remove all the GMP, and the eluting the ApUpGp with 0.5 M TEAB. The trimer was concentrated and desalted as before. The 5′ phosphorylation of ApUpGp was carried out in a 3.5-mL reaction containing 5 mM ApUpGp, 7.5 mM ATP, buffer B (pH 7.5), and 25 units/mL *PseT* 1 polynucleotide kinase. After incubation for 8 h at 37 °C, the reaction was purified on a 3-mL DEAE-Sephadex A-25 column. A total of 11.3 μmol of pApUpGp (65%) was recovered after desalting.

pGpApUp was prepared in two different ways. The first involved incubating 6.5 mM GpA, 40 mM UDP, 40 μg/mL ribonuclease A, buffer A, and 4.0 units/mL polynucleotide phosphorylase in a 2-mL reaction for 24 h at 37 °C. GpApUp (6.4 μmol, 50%) was recovered from a 3-mL DEAE-Sephadex A-25 column. pGpApUp was prepared in a 6.4-mL reaction by incubating 1.0 mM GpApUp, 3 mM ATP, buffer B (pH 7.5), and 15.6 units/mL *PseT* 1 polynucleotide kinase for 4 h at 37 °C. pGpApUp (6 μmol, 90%) was recovered from a 10-mL DEAE-Sephadex A-25 column.

The second method for the synthesis of pGpApUp used a sequential procedure that avoided one of the purification steps. A 4-mL reaction containing 5 mM GpA, 0.14 mM ATP, 16 mM creatine phosphate, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 M Tris-HCl, pH 8.3, 60 units/mL creatine phosphokinase, and 660 units/mL polynucleotide kinase was incubated for 12 h at 37 °C. After a brief heating at 90 °C, the reaction was evaporated to dryness. The reaction was then made 20 mM UDP, 40 μg/mL RNase A, and 4.0 units/mL polynucleotide phosphorylase (4-mL total volume) and incubated for 24 h at 37 °C. The presence of an ATP regenerating system in the first step prevented the accumulation of ADP, which would have interfered in the polynucleotide phosphorylase reaction. pGpApUp (8.4 μmol, 42%) was purified on a 10-mL DEAE-Sephadex column.

ApUpGpUp was prepared in a 5-mL reaction containing 1 mM ApUpG, 2 mM pUp, 4.5 mM ATP, buffer B (pH 7.6), 10% (v/v) dimethyl sulfoxide, and 90 μg/mL RNA ligase. After 12 h at 25 °C, the reaction was purified by paper chromatography in solvent A. This procedure yielded 2 μmol of ApUpGpUp (40% of the input ApUpG).

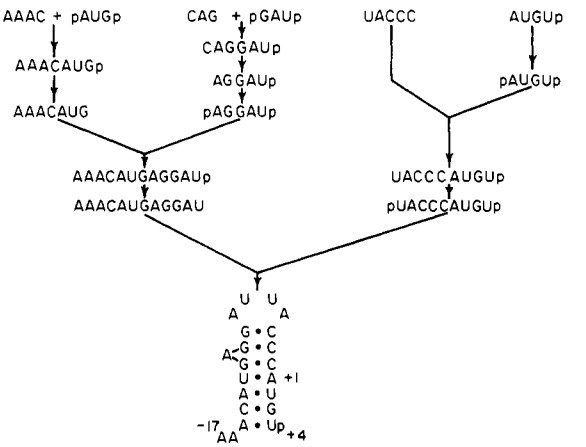


FIGURE 1: Synthetic scheme for the 21-nucleotide R17 coat protein binding fragment. The first nucleotide of the replicase gene initiation codon is designated +1.

Tritium-labeled acceptor molecules ApApApC, $UpA(pC)_3$, ApUpG, and CpApG were prepared from ApApA, $UpA(pC)_2$, ApU, CpA, and the corresponding $^3H$-labeled nucleoside diphosphates with polynucleotide phosphorylase (Uhlenbeck & Cameron, 1977). 5′-$^{32}P$-Labeled donor molecules were prepared with [γ-$^{32}P$]ATP and *PseT* 1 polynucleotide kinase (Cameron et al., 1978). These labeled oligomers were added to their nonlabeled counterparts to obtain the desired specific activity in trial reactions.

## Results

The general scheme for the synthesis of the 21-nucleotide coat protein binding fragment is shown in Figure 1. A branched synthetic protocol allows the use of approximately equivalent amounts of the six starting oligomers and is easier to adapt for the synthesis of variant sequences. The particular combination of starting oligomers is dictated by the efficiency of their synthesis with polynucleotide phosphorylase and the expected yields of RNA ligase reactions based on previous studies with model compounds (England & Uhlenbeck, 1978). The synthesis of each half-molecule will be described separately, followed by the synthesis, characterization, and assay of the 21-mer.

*Synthesis of 5′ Half-Molecule ApApApCpApUpGpApGpGpApU.* The equimolar joining reaction between [*Cyd*-$^3H$](Ap)$_3$C and [5′-$^{32}P$]pApUpGp is shown in Figure 2. The reaction conditions are quite similar to those described previously for the synthesis of $(Ap)_3Cp(Up)_5$ (Uhlenbeck & Cameron, 1977). The presence of 3′-phosphate on the donor molecule prevents self-addition or multiple additions of donor to the acceptor. The unique product $(Ap)_3CpApUpGp$ is made in about 80% yield. Its identity could be confirmed by the fact that total nuclease digestion of the product of the trial reaction gave [$^3H$,$^{32}P$]CMP. For the preparative reaction, the reaction in Figure 2 was scaled up 90-fold without radiolabeled oligomers. After the reaction was complete, it was heated for 3 min at 90 °C to inactivate the RNA ligase and then incubated with 10 μg/mL bacterial alkaline phosphatase for 20 h at 37 °C to remove the 3′-terminal phosphate. The heptamer was purified on a 10-mL DEAE-Sephadex A-25 column eluted with a 200-mL linear gradient from 0.1 to 0.8 M NaCl. The pooled $(Ap)_3CpApUpG$ (5.7 μmol, 63%) was desalted twice on Bio-Gel P2.

The synthesis of pApGpGpApUp was carried out in three steps. CpApG was first joined with pGpApUp to form CpApGpGpApUp. The purified hexamer was reacted with ribonuclease A to remove the 5′-terminal CMP and then with

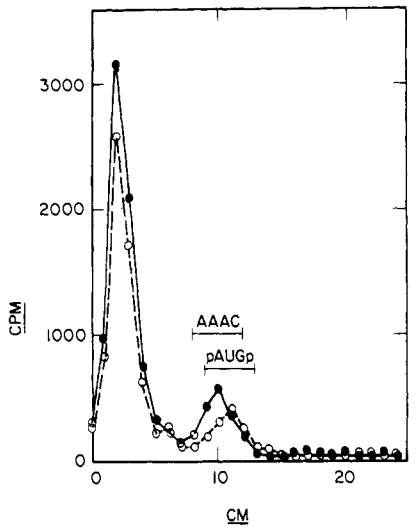


FIGURE 2: Synthesis of ApApApCpApUpGp. The 100-$\mu$L reaction contained 1 mM [*Cyd*-$^3$H](Ap)$_3$C (0.85 Ci/mol), 1 mM [5′-$^{32}$P]-pApUpGp (0.45 Ci/mol), 1.4 mM ATP, buffer B (pH 8.3), and 70 $\mu$g/mL RNA ligase. After incubation at 10 °C for 48 h, the $^{32}$P-labeled (○) and $^3$H-labeled (●) reaction products were separated by descending paper chromatography in solvent C.

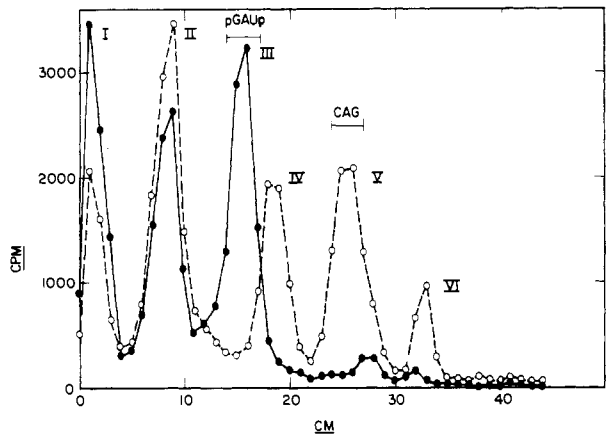


FIGURE 3: Synthesis of CpApGpGpApUp. The 25-$\mu$L reaction contained 0.1 mM [*Guo*-$^3$H]CpApG (18 Ci/mol), 0.1 mM [5′-$^{32}$P]pGpApUp (6 Ci/mol), 0.4 mM ATP, buffer B (pH 8.3), and 160 $\mu$g/mL RNA ligase. After incubation at 10 °C for 24 h, the reaction products were separated by descending paper chromatography in solvent B. Open circles and dotted lines represent $^3$H label and closed circles and solid lines represent $^{32}$P label. Peak VI is [$^3$H]GMP present as an impurity in the [*Guo*-$^3$H]CpApG starting material.

polynucleotide kinase and ATP to introduce the 5′-terminal phosphate. The equimolar joining of [*Guo*-$^3$H]CpApG and [5′-$^{32}$P]pGpApUp run under standard conditions and analyzed by paper chromatography is shown in Figure 3. Not only is the expected [$^3$H,$^{32}$P]CpApGpGpApUp obtained (peak II) but also two additional oligomers, CpApGpGpApGpApUp (peak I) and CpApGpUp (peak III), are made. This reaction is very similar to the equimolar joining of ApCpG and pGpApUp analyzed in detail elsewhere (Krug & Uhlenbeck, 1982). The additional two peaks are the result of the reversal of the last step of the RNA ligase reaction. After the forward reaction produces CpApGpGpApUp and AMP, the same two molecules react in a reverse reaction to produce CpApGpGpA and A-5′-pp-5′-Up. Peak I is then the result of joining CpApGpGpA with pGpApUp in the ATP-dependent forward reaction, and peak III is the result of joining CpApG with A-5′-pp-5′-Up in the ATP-independent forward reaction. These side reactions greatly reduce the yield of the desired product. Although several attempts to suppress the reverse

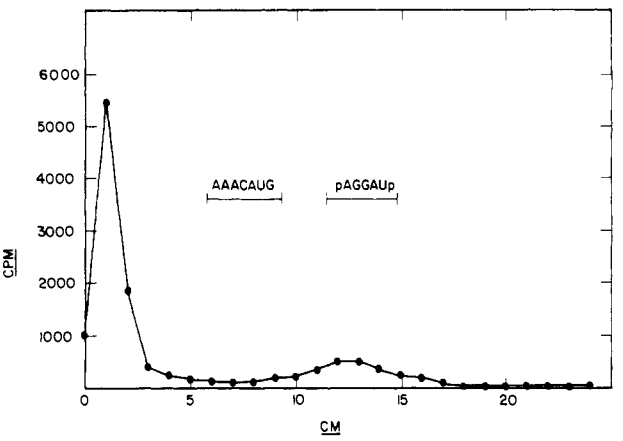


FIGURE 4: Synthesis of 5′ half-molecule. The 25-$\mu$L reaction contained 0.2 mM (Ap)$_3$CpApUpG, 0.1 mM [5′-$^{32}$P]pApGpGpApUp (14 Ci/mol), 2.5 mM ATP, buffer B (pH 8.3), and 160 $\mu$g/mL RNA ligase. After incubation at 16 °C for 8 h, the reaction products were separated by descending paper chromatography in solvent C.

reaction were made (Krug & Uhlenbeck, 1982), only small improvements in the yield could be obtained. The optimal synthesis conditions used in a 5-mL preparative reaction were 1 mM CpApG, 0.5 mM pGpApUp, 2 mM ATP, and 140 $\mu$g/mL RNA ligase in buffer B (pH 8.3) incubated 8 h at 16 °C. The reaction products were purified by paper chromatography with solvent B. The 0.9 $\mu$mol of CpApGpGpApUp that was recovered represents a 35% yield with respect to the limiting pGpApUp donor. However, under these reaction conditions, comparatively little CApGpGpApGpApUp (0.14 $\mu$mol) had accumulated so that 1.1 $\mu$mol (45%) of unreacted pGpApUp and 2.5 $\mu$mol (50%) of unreacted CpApG could be recovered for further use. If the reaction had been incubated longer or more enzyme had been used, the amount of CpApGpGpApUp recovered would have remained the same and more of peaks I and III would have been obtained.

The CpApGpGpApUp recovered from paper was purified on Bio-Gel P2 to remove an inhibitor of polynucleotide kinase. The removal of the 5′-terminal CMP and the subsequent 5′ phosphorylation of the pentamer were carried out sequentially in the same reaction mixture. A 50-$\mu$L reaction containing 0.63 mM CpApGpGpApUp and 12.5 $\mu$g/mL RNase A was incubated in buffer B (pH 8.3) at 37 °C for 60 min. Then 1.4 $\mu$mol of ATP and 140 units of *PseT* 1 polynucleotide kinase were added in 140 $\mu$L and incubated for 2 h at 37 °C. The pApGpGpApUp (0.3 $\mu$mol, 85%) was purified by paper chromatography in solvent B.

A chromatogram of the reaction joining the pentamer donor [5′-$^{32}$P]pApGpGpApUp and the heptamer acceptor (Ap)$_3$CpApUpG to form the 5′ half-molecule is shown in Figure 4. Since maximal incorporation of the more valuable donor was desirable, a 2-fold excess of acceptor was used. Approximately 75% of the $^{32}$P label is incorporated into a longer product. When the same reaction was analyzed on an acrylamide gel, the labeled product migrated as a single band with a mobility similar to a (Cp)$_{11}$Gp marker. Virtually no material longer than the correct product was detected on the gel, suggesting that little reversal of the RNA ligase reaction occurred in this reaction. When the $^{32}$P-labeled product was eluted from the chromatogram in Figure 4 and digested with nucleases, the only product obtained was [3′-$^{32}$P]GMP, supporting the identity of the dodecamer. For a preparative synthesis, the trial reaction was scaled up 200-fold without the radioactive donor. After the reaction was complete, it was incubated at 90 °C for 5 min to inactivate the RNA ligase.

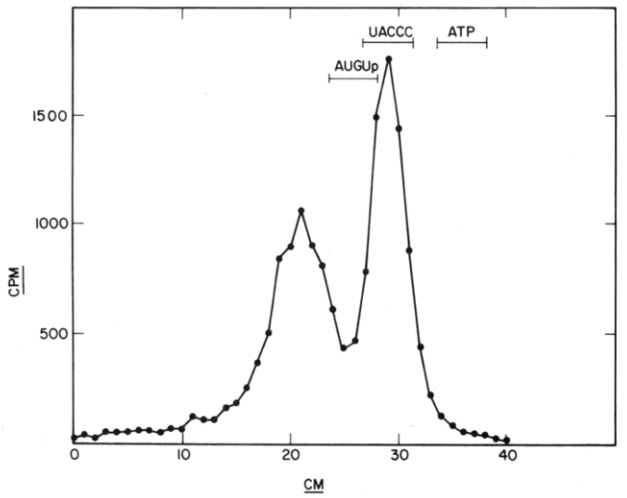


FIGURE 5: Synthesis of 3′ half-molecule. The 20-μL reaction contained 1 mM ApUpGpUp, 2.3 mM ATP, buffer B (pH 8.3), and 115 μg/mL *PseT* 1 polynucleotide kinase. After incubation at 37 °C for 4 h, 5 μL of 2 mM [*Cyd*-$^3$H]UpApCpCpC (2 Ci/mol), 9 mM ATP, and 1 mg/mL RNA ligase was added. After further incubation at 25 °C for 12 h, the reaction products were separated by descending paper chromatography in solvent C.

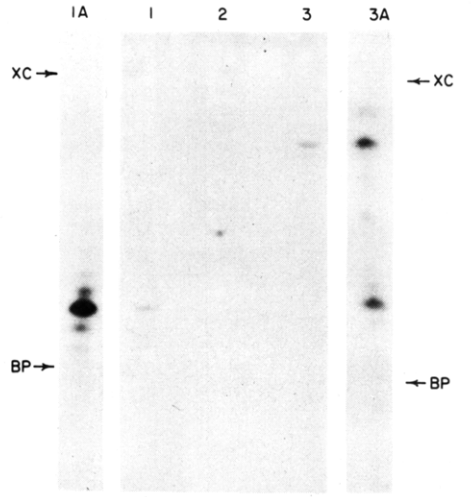


FIGURE 6: Synthesis of 21-nucleotide fragment. The joining of the two half-molecules is analyzed by denaturing gel electrophoresis. The central panel is the gel stained with Stains-All, and the two outside panels 1A and 3A are the autoradiograms of the corresponding lanes of the gel. Lane 1 contained 0.4 nmol of 5′-$^{32}$P-labeled 3′ half-molecule (0.1 Ci/mmol). Lane 2 contained 0.26 nmol of the 5′ half-molecule. Lane 3 contained a mixture of 10 μL from the preparative reaction and 2 μL from the analytical reaction.

Then 100 μg of alkaline phosphatase was added and incubated for 4 h at 37 °C to remove the 3′-terminal phosphate. The 5′ half-dodecamer was purified by paper chromatography in solvent C. The recovery of $(Ap)_3CpApUpGpApGpGpApU$ (0.13 μmol) corresponds to a 65% yield with respect to the limiting pentamer donor.

*Synthesis of 3′ Half-Molecule pUpAp(Cp)$_3$ApUpGpUp.* The phosphorylation of ApUpGpUp and its subsequent joining to UpA(pC)$_3$ were carried out sequentially in the same reaction mixture to avoid losses in the isolation of pApUpGpUp. A 2-fold molar excess of ApUpGpUp was employed to optimize the use of the more valuable UpA(pC)$_3$. The initial intention had been to purify the nonamer product with a free 5′-hydroxyl so the similar sequential action of kinase and ligase could be used for the synthesis of the 21-mer. However while doing trial reactions, it was inadvertently discovered that if the kinase was not inactivated after the first step, a 5′-phosphorylated nonamer resulted. In Figure 5 a trial reaction of this multistep procedure is shown. Approximately 40% of the [$^3$H]UpA(pC)$_3$ is converted to the slower moving nonamer. A preparative reaction was carried out under very similar conditions. A 1.8-mL reaction contained 0.45 mM ApUpGpUp, 1 mM ATP, and 200 units/mL *PseT* 1 kinase. After 4 h at 37 °C, 0.4 μmol of UpA(pC)$_3$, 1.8 μmol of ATP, and 200 μg of RNA ligase were added in 200 μL and further incubated for 8 h at 25 °C. The 3′ half-molecule pUpAp(Cp)$_3$ApUpGpUp (0.14 μmol, 35%) was purified by paper chromatography with solvent C.

Several lines of evidence suggest that the product of the reaction was entirely the 5′-phosphorylated 3′ half-molecule and did not contain products resulting from the 5′ phosphorylation and self-addition of UpA(pC)$_3$. First, electrophoresis of the 3′ half-molecule on a polyacrylamide gel showed only a single stained band migrating close to a nonamer marker (Figure 6). Contaminating cyclic decamer formed by two UpA(pC)$_3$ molecules would migrate more rapidly (de Haseth & Uhlenbeck, 1980). Second, the product could not be phosphorylated with [γ-$^{32}$P]ATP and polynucleotide kinase until it first had been treated with alkaline phosphatase. This confirms the presence of the 5′-terminal phosphate. Total hydrolysis of the 5′-$^{32}$P-labeled oligomer gave the expected [5′-$^{32}$P]pUp while treatment with ribonuclease T$_1$ resulted in an oligomer one nucleotide shorter on a polyacrylamide gel as expected by the sequence. Finally, the 3′-terminal nucleotide of the oligomer was identified by reacting the dephosphorylated oligomer with [5′-$^{32}$P]pCp and RNA ligase and purifying the radioactive product. Hydrolysis of this oligomer with a mixture of ribonucleases gave only [3′-$^{32}$P]UMP. Any linear decamer resulting from the self-addition of UpA(pC)$_3$ would have resulted in [3′-$^{32}$P]CMP by this protocol. Thus, despite the possibility for alternate products, the three-step synthesis of the 5′-phosphorylated 3′ half-molecule was successful. In cases where 5′-$^{32}$P-labeled 3′ half-molecule was desired, the polynucleotide kinase was inactivated after the first step, and the nonamer was purified with a 5′-hydroxyl. The 5′-$^{32}$P label was then introduced by a separate *PseT* 1 polynucleotide kinase reaction.

*Synthesis of 21-Nucleotide Fragment.* Two separate RNA ligase reactions joining the nonamer donor and the dodecamer acceptor were carried out. A 500-μL preparative reaction containing 25 μM dodecamer, 25 μM nonamer, 2.3 mM ATP, and 240 μg/mL RNA ligase was incubated in buffer B (pH 8.3) for 12 h at 16 °C. The high RNA ligase concentration required for good yields suggests that, despite their potential for base pairing, the two half-molecules are not associated during the reactions. Much lower RNA ligase concentrations (3 μg/mL) were needed to join the two half-molecules of tRNA$^{Phe}$ (Bruce & Uhlenbeck, 1982). Attempts to raise the reaction yields at lower enzyme concentrations by increasing oligomer concentrations or decreasing the temperature were not successful. A second 6-μL analytical reaction containing 0.5 μM 5′-$^{32}$P-labeled nonamer (140 Ci/mmol), 43 μM dodecamer, and 1.6 mM ATP was incubated in the same buffer under the same conditions to provide internally labeled 21-mer of high specific activity. Aliquots of the preparative and analytical reactions were combined and analyzed by denaturing gel electrophoresis in Figure 6. The stained gel shows that essentially all of the donor and the acceptor in the preparative reaction are converted to a single band that migrates slightly faster than xylene cyanole as expected for a 21-nucleotide fragment. In this size range, the resolution of the gel is sufficient to distinguish oligomers one nucleotide longer or shorter. Thus, a single major oligomer product was obtained from the preparative ligase reaction. The autoradiogram of

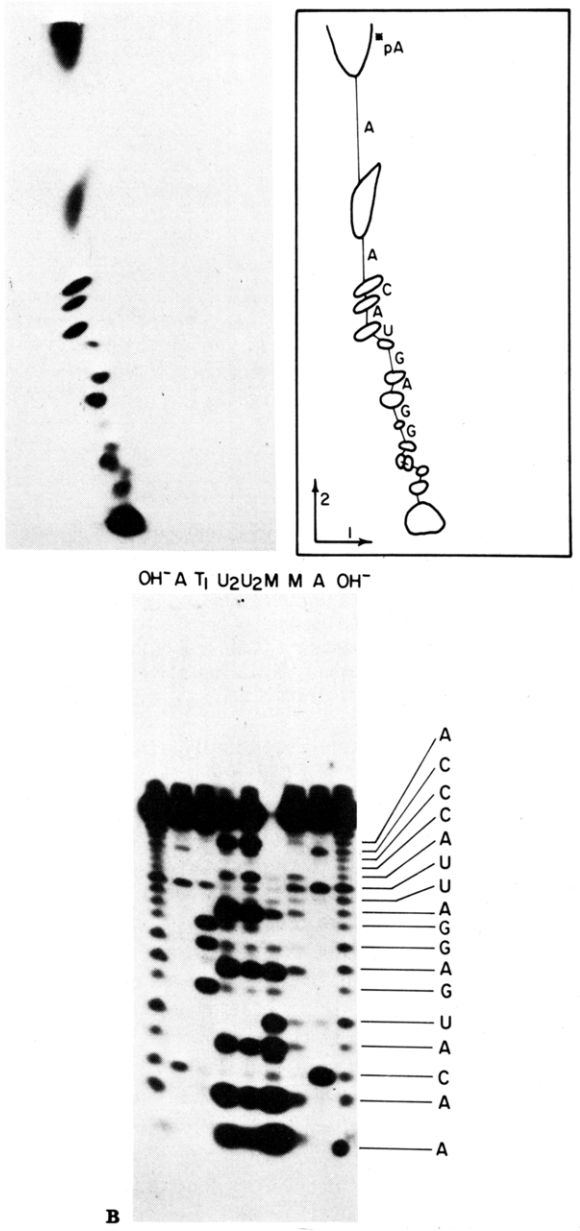


FIGURE 7: Sequence analysis of 21-nucleotide synthetic fragment. Panel A is the autoradiogram of a partial snake venom phosphodiesterase digest of 5′-$^{32}$P-labeled 21-nucleotide fragment analyzed by two-dimensional chromatography. The first dimension is electrophoresis at pH 3.5 on cellulose–acetate, and the second dimension is homochromatography on a 40 × 20 cm DEAE-cellulose thin-layer plate. The deduced sequence is presented in the diagram at the right. Panel B is the autoradiogram of gel electrophoretic analysis of partial enzymatic and base-catalyzed digestions of 5′-$^{32}$P-labeled 21-nucleotide fragment. The two outside lanes marked $OH^-$ are partial base hydrolyses. The letter designations for the remaining lanes refer to the ribonuclease that was used to generate the digest in these lanes. Two different times of incubation are presented for each nuclease except ribonuclease $T_1$ to ensure a proper distribution of partial digestion products.

the gel, which analyzes the products of the analytical reaction, also shows a major oligomer product in somewhat lower yield that precisely comigrates with the preparative product. An overexposed autoradiogram of the gel shows some material at even higher molecular weight that may be the result of the reversal of RNA ligase. When the main oligomer product was cut out of the gel, eluted, and hydrolyzed with mixed endonucleases, only the expected mononucleotide [3′-$^{32}$P]UMP was obtained. When the same product was treated with bacterial alkaline phosphatase to remove the 3′-terminal phosphate and

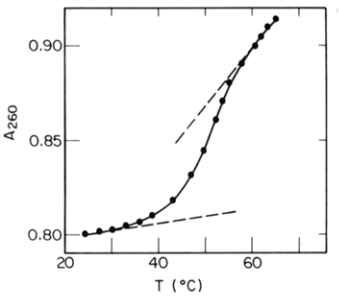


FIGURE 8: Absorbance–temperature profile of 21-nucleotide fragment in 10 mM sodium phosphate (pH 7.0) and 1 mM $Na_2EDTA$. Dotted lines indicate the extrapolated absorbance of helical and single-stranded molecules (Uhlenbeck et al., 1973).

then with snake venom exonuclease to generate 5′-monophosphates, only the expected [5′-$^{32}$P]UMP was obtained. These experiments show that the correct internucleotide linkage was formed between the donor and acceptor.

The remainder of the preparative reaction was ethanol precipitated and purified by gel electrophoresis as described under Materials and Methods. While only 3.3 nmol (25%) of the 21-nucleotide fragment was obtained after elution from the gel, the poor recovery is apparently due to the elution procedure since no unreacted half-molecules were seen on the gel by ultraviolet shadowing.

The gel-purified 21-nucleotide fragment was phosphorylated with [$\gamma$-$^{32}$P]ATP and *PseT* 1 polynucleotide kinase, and the reaction was analyzed on analytical 20% polyacrylamide gels containing 7 M urea and either Tris–borate (pH 8.3) (Donis-Keller et al., 1977) or sodium citrate (pH 3.5) (deWachter & Fiers, 1971). In both cases a single sharp band was observed after autoradiography, further confirming the purity of the fragment. Labeling of the 3′ terminus of the dephosphorylated fragment with [5′-$^{32}$P]pCp and RNA ligase also resulted in a single radioactive band. The 3′ and 5′ terminally labeled fragments were eluted from the gels and hydrolyzed with ribonucleases to confirm the identity of the terminal nucleotide. As expected, [5′-$^{32}$P]pAp was obtained with the 5′-$^{32}$P-labeled fragment and [3′-$^{32}$P]UMP was obtained with the pCp-labeled fragment. Since no other labeled nucleotides were found, this result confirms the purity of the product.

The 5′-$^{32}$P-labeled fragment was also used to carry out nucleotide sequence analysis. The result of "wandering spot" nucleotide sequence analysis (Silberklang et al., 1977) is presented in Figure 7A. The sequence of the 5′-terminal ten nucleotides was determined in this experiment. Of particular importance is the absence of additional minor spots, which demonstrates that the oligomer is not contaminated by other sequences. In Figure 7B, the sequence of the 5′-$^{32}$P-labeled fragment is determined by gel electrophoresis with sequence specific nucleases to obtain partial digestions (Koper-Zwarthoff et al., 1977; Lockard et al., 1978). In this case, the sequence of the 5′-terminal 18 nucleotides was confirmed.

*Secondary Structure and Protein Binding.* Since the 21-nucleotide fragment is expected to form a hairpin loop closed by a stem of seven base pairs, an absorbance–temperature profile was determined in 10 mM sodium phosphate buffer (Figure 8). A single transition with a breadth characteristic of short oligomer helices occurs between 40 and 55 °C. A strong single-stranded unstacking component is observed above 55 °C. The 10% hypochromicity is approximately what is expected for an oligomer that is only two-thirds helical and rich in GC pairs. The midpoint of the transition is 49 °C, which agrees very well with the value of 48 °C obtained at the same ionic strength for the corresponding helix in the

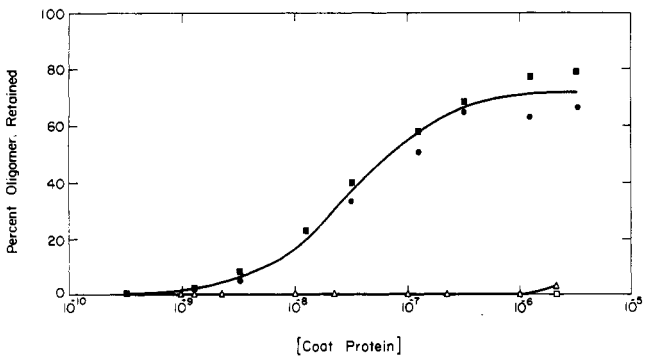


FIGURE 9: Binding of R17 coat protein to 5′-$^{32}$P-labeled 21-nucleotide synthetic fragment (■), 5′-$^{32}$P-labeled 59-nucleotide natural fragment (●), 5′-labeled half-molecule (△), and 5′-$^{32}$P-labeled 3′ half-molecule (□) in the presence of 10 μg/mL tRNA carrier, 80 mM KCl, 10 mM $MgCl_2$, and 100 mM Tris-HCl, pH 7.5, at 20 °C. The calculated first-order binding curve, with $K = 2 \times 10^7$ $M^{-1}$ and 70% retention efficiency, is given by the solid line.

59-nucleotide fragment of MS2 RNA (Gralla et al., 1974).

A nitrocellulose filter assay (V. Cameron, J. Carey, P. L. de Haseth, and O. C. Uhlenbeck, unpublished results) was used to measure the binding of a low concentration of the 5′-$^{32}$P-labeled 21-nucleotide fragment to varying concentrations of R17 coat protein. As can be seen in Figure 9, a binding curve is observed with a midpoint at about 0.1 μM coat protein. At the highest protein concentration, nearly 80% of the oligomer is retained on the filter. If one assumes that each protein can bind one RNA fragment and that the retention efficiency of the complex on nitrocellulose filters is 70%, a dissociation constant of $2 \times 10^7$ $M^{-1}$ can be calculated. Neither of the two 5′-$^{32}$P half-molecules are able to bind the coat protein. Although an equimolar mixture of the two half-molecules showed no detectable binding, their concentration in the binding assay was too low for the two oligomers to be base paired.

Also shown in Figure 9 is the binding of the 5′-$^{32}$P-labeled 59-nucleotide fragment of R17 RNA isolated by coat protein protection. The binding curve of the natural fragment is identical with that of the synthetic fragment. Since it had previously been shown that the 59-nucleotide fragment binds coat protein just as well as intact R17 RNA (Berzin et al., 1978), we can conclude that the 21-nucleotide synthetic fragment has full biological activity.

## Discussion

The efficiency with which T4 RNA ligase can join two oligoribonucleotides (Gumport & Uhlenbeck, 1981) suggests that relatively long fragments of RNA can be synthesized by totally enzymatic methods. Since the synthetic scheme in this work involves five different RNA ligase reactions to join six short oligomers into the final 21-nucleotide fragment, it is a good test for the feasibility of a lengthy synthesis. Although optimal conditions for each ligase reaction varied somewhat, reaction yields were generally quite high, varying from 35 to greater than 90%. Since not all the material from each step was used in the following step and reaction conditions were generally adjusted to make optimal use of the most valuable component, it is not meaningful to calculate a percentage yield for the overall synthesis. However, in order to recover 1 nmol of 21-mer, we would require from 11 to 40 nmol of each of the six starting oligomers. The largest losses were encountered in the synthesis of CAGGAUp, where the reversal of the RNA ligase reaction substantially decreased the yield of the correct product. The development of a better 3′-terminal donor blocking group should alleviate this problem. We found little evidence for RNA ligase reversal in any of the other reactions.

A second difficulty in an extended synthesis is the losses that are encountered in the purification of intermediates. Much less material was obtained after purification than was expected on the basis of the yield calculated by the incorporation of radioactivity. This problem was especially great as the size of the oligomer increased. Although this problem can be offset to some extent by carrying out several sequential enzyme reactions before purification, it remains a major problem in preparing very long sequences. Recently developed high-resolution chromatography systems to separate unprotected oligomers are expected to be helpful (McFarland & Borer, 1979; McLaughlin et al., 1981; Vandenberghe, 1980).

The purity of the 21-nucleotide product of the synthesis was demonstrated in several ways. First, the product migrated as a single band on two different high-resolution polyacrylamide gel systems. Second, no minor components were observed by the wandering spot sequencing procedure. Third, the formation of a unique and correct Up(3′→5′)U internucleotide linkage in the last RNA ligase step was confirmed. Finally, determination of the 3′ and 5′ termini of the molecule resulted in a unique nucleotide, indicating the lack of contamination by other oligomers.

The binding properties of the 21-nucleotide synthetic fragment to R17 coat protein were found to be identical with those of a 59-nucleotide fragment isolated from R17 RNA. This assay confirms that the synthetic protocol resulted in a molecule with full biological activity. The specificity of this interaction is quite high. R17 coat protein does not bind appreciably to other regions of R17 RNA or to several other high and low molecular weight RNA molecules (Steitz, 1974; V. Cameron, J. Carey, P. L. de Haseth, and O. C. Uhlenbeck, unpublished results). Synthetic control over the coat protein binding sequence should now permit the construction of variant sequences that will allow the identification of the nucleotides responsible for the specific protein–RNA interaction.

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# Characterization of the Collagens Synthesized by Chinese Hamster Ovary Cells. Effect of Colcemid and Dibutyryladenosine Cyclic Monophosphate†

Hardy Limeback,* Jaro Sodek, and Jane Aubin

ABSTRACT: The collagens synthesized by Chinese hamster ovary cells have been isolated and characterized. Although these cells produce very small amounts of collagen, at least five distinct collagenous chains could be identified from radiolabeled media and cell extracts after limited pepsin digestion. Two chains were characterized as $\alpha_1$(V) and $\alpha_2$(V), based on electrophoretic mobility, resistance to vertebrate collagenase, chromatographic properties on carboxymethylcellulose, and cyanogen bromide peptide patterns. Two smaller collagenous proteins ($M_r$ 34000 and 37000) were also isolated by carboxymethylcellulose chromatography and characterized by cyanogen bromide digestion patterns. These collagens showed similarities to type IV collagen fragments but may be unique to Chinese hamster ovary cells. A colcemid-resistant mutant of Chinese hamster ovary cells designated CM$^R$795 [Ling, V., Aubin, J. E., Chase, A., & Sarangi, F. (1979) *Cell (Cambridge, Mass.) 18*, 423–430] was found to synthesize the same collagen chains but in different proportions. In the wild-type cells colcemid (0.05–0.1 μg/mL) reduced the amount of type V collagen in the culture media but had little effect on the other collagen type, whereas the type V collagen reduction was less pronounced in the CM$^R$795 cells treated with the same concentrations of colcemid. Dibutyryladenosine cyclic monophosphate caused a fibroblast-like "reverse transformation" of the Chinese hamster ovary cells similar to that described previously [Hsie, A. W., & Puck, T. T. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 358–361]. However, collagen synthesis was increased only slightly. Furthermore, no apparent alteration in the types of collagens synthesized was detected.

Various cell types have been used to study the biosynthesis and molecular heterogeneity of collagen. Usually the collagen synthetic pattern is characteristic of the cell type examined and of the tissue of origin. For example, fibroblasts and smooth muscle cells synthesize type I and type III collagens whereas chondrocytes synthesize type II collagen [see Bornstein & Sage (1980) for recent review]. However, the cellular origin of basement membrane collagens, type IV (Kefalides, 1975) and possibly type V[1] (Roll et al., 1980), is unknown; they may be products of epithelial cells, connective tissue cells, or both. Some fibroblasts (Herrmann et al., 1980; Gay et al., 1980) and even bone cells (Aubin et al., 1982) are capable of synthesizing type V collagen in vitro, but synthesis of type

† From the Medical Research Council Group in Periodontal Physiology, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8. *Received January 5, 1982.* This work was supported by the Medical Research Council of Canada and by a grant from the Faculty of Dentistry, University of Toronto.

[1] The nomenclature used for type V collagen chains is described by Bornstein & Sage (1980). α A and α B have been designated $\alpha_2$(V) and $\alpha_1$(V), respectively.

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